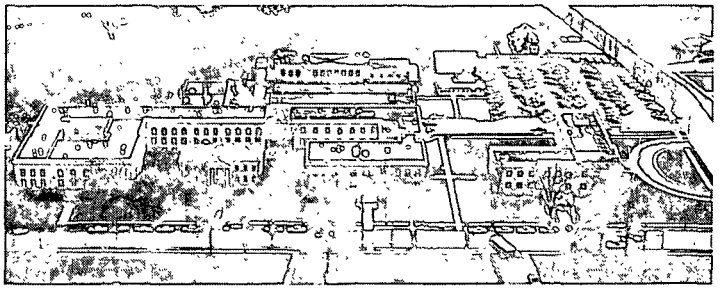

THE INSTITUTE OF PAPER CHEMISTRY, APPLETON, WISCONSIN

IPC TECHNICAL PAPER SERIES

NUMBER 326

TRIGLYCERIDES IN EMBRYOGENIC CONIFER CALLI: A COMPARISON WITH ZYGOTIC EMBRYOS

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FEBRUARY, 1989

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This manuscript is based on results obtained in IPC research and has been submitted for consideration for publication in Plant Cell Reports

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ABSTRACT

Triglycerides in developing zygotic embryos of Norway spruce and loblolly pine were found to accumulate continuously during the course of development, comprising nearly 50% of the fresh weight of a mature embryo. Embryogenic calli of these two species contained dramatically lower levels of triglycerides. Low levels of triglycerides may be related to difficulties in the late development and germination of conifer somatic embryos. Abscisic acid treatments promoted both embryo production and triglyceride accumulation in Norway spruce cultures. Levels of triglycerides were measured biochemically in this study, rather than by the less precise gravimetric procedures. A method used to determine triglyceride levels in human serum, commercially available in kit form, was adapted for use with plant tissues.

INTRODUCTION

Ultrastructural examinations have revealed several differences between developing zygotic and somatic conifer embryos. Characteristic, undifferentiated plastids are typically found in somatic embryos during early stages of development (Feirer and Becwar, submitted for publication). Also notable were differences in the number of lipid bodies present in the embryos, appearing to be significantly more abundant in zygotic embryos. Lipid bodies (also termed oil droplets or oleosomes) are composed primarily of triglycerides. The biosynthesis of the triglycerides present in the lipid bodies has been extensively studied, especially in oil-rich seeds where the triglycerides represent major deposits of storage material

(Gure, M.I., 1980; Harwood, 1980; Styme and Stobart, 1987). Storage lipids comprise 30 to 50% of the dry weight of conifer seeds, serving as reserves required during the germination process (Ching 1963, 1966; Konar, 1958). The mere abundance of storage lipids in conifer seeds suggests a major role for triglycerides during the development and subsequent germination of embryos. A deficiency of lipids in somatic embryos might then hinder their development and germination. An objective of this study was to quantify levels of triglycerides in embryogenic calli and somatic embryos of two conifer species, and to compare levels found in cultured tissues to those measured in developing zygotic embryos.

While levels of "total lipids", determined gravimetrically, and relative ratios of free fatty acids are often measured in plant tissues, a more accurate biochemical assay was employed in this study. The method described, commonly used to determine triglyceride levels in human serum, was adapted for the measurement of triglycerides in plant tissues.

MATERIALS AND METHODS

Plant tissues

Developing embryos were obtained from Norway spruce (Picea abies (L.) Karst.) and loblolly pine (Pinus taeda L.) cones collected at intervals throughout the 1988 summer growing season. Norway spruce cones were collected from 2 clones grown near Syracuse, NY. Loblolly pine cones were from 2 clones grown in seed orchards near Summerville, SC. Within a species, clonal differences in triglyceride levels were found to be nonsignificant and data from the clones were subsequently summed. Since seed and embryo development within a cone were found to be asynchronous, tissues were not collected strictly on the basis of calendar date. Rather, immature embryos dissected from the cones were assigned to several arbitrary stages of development: stage 1 and 2 represented precotyledonary embryos, cotyledonary primordia were just visible in stage 3, and stage 4 immature embryos had well defined and separated cotyledons. Stage 5 embryos, judged to be mature and fully developed, were isolated from cones that were beginning to brown and open. Isolated embryos and female gametophyte tissue, free of embryo, integuments and seedcoat, were collected and stored at -70 °C until analysis.

Embryogenic calli of Norway spruce and loblolly pine were initiated and maintained as previously described (Becwar et al., 1987; 1988). Abscisic acid (ABA) was filter sterilized before addition to

autoclaved medium. Carrot (Daucus carota L.) callus was initiated and maintained on modified MS medium (Murashige and Skoog, 1962) containing 2,4-D (1.0 mg/l) and kinetin (0.02 mg/l).

Triglyceride determination

During the course of this work, the necessity of a convenient, reliable assay for the measurement of storage lipids (triglycerides) became apparent. Since an assay suitable for small amounts of plant tissues was not found in the literature, a technique designed for the clinical determination of triglycerides in blood was adapted for use. The assay is based on a diagnostic kit procedure available from Sigma Chemical Company (anonymous, 1984) for the measurement of triglycerides in serum or plasma. The method, described in this report, has been modified to better suit plant tissues. Briefly, triglycerides are purified, saponified and the resulting glycerol is quantified colorimetrically. A more detailed presentation complete with a list of reagents and equipment suppliers is available as an IPC Technical Paper Series (Conkey and Feirer, 1988).

Triglyceride extraction

Due to the sensitivity of the triglyceride assay, only small amounts of tissue are required. When using mature seed, as little as 5 mg of tissue is required, although 50-200 mg of callus tissues are necessary. After collection into tared 1.5 ml microfuge tubes and fresh weights are determined, samples may be stored at -70 °C until needed. A 100 µl volume of isopropanol is added per microfuge tube and the tissue homogenized using a pestle fitted to the microfuge tube (Kontes Inc). The tube volume is brought to one ml with isopropanol. The extraction is continued by placing the tubes on a shaker for 15 min. Finally, the tubes are centrifuged for 5 min. in an Eppendorf microfuge.

Triglyceride purification

An 800 µl volume of the supernatant is transferred to a vial containing 0.8 g of activated alumina purifier and 1.8 ml of isopropanol. The vials are shaken for a minimum of five minutes before the alumina is sedimented by a brief centrifugation in a table top centrifuge.

Color development

An 800 µl volume of the "purified" supernatant is transferred to a small polypropylene test tube with a snap cap. It is important that this sample and the reagents are well mixed after each of the following additions. First, 200 µl of 1N KOH is added and the tubes held in a 60 °C water bath for 5 min. After cooling to room temperature, 200 µl of a sodium periodate solution is added to each tube. After exactly 10 min., 1.2 ml of color reagent is added and the tubes returned to the 60 °C water bath for 30 minutes. The resulting color is quite stable but Sigma recommends that the absorbance be measured within 20 minutes.

Triglyceride quantification

The contents of each tube are transferred to a 4.5 ml cuvette and the A_{410} measured. Standards, prepared from triolein, are carried through the entire procedure. One ml volumes of isopropanol containing 0, 126, 375 and 750 µg of triolein provided a linear standard curve suited to the triglyceride levels encountered in our tissues. A linear regression based on the standards results in a correlation coefficient greater than 0.99 and is used to convert the unknown absorbance to g/ml triglyceride.

RESULTS AND DISCUSSION

Stage 1 zygotic embryos were found to be too small for isolation. Consequently, the values illustrated for stage 1 represent triglycerides in the immature embryo plus the surrounding female gametophyte. Triglycerides accumulated nearly continuously over the course of both Norway spruce and loblolly pine zygotic embryo development (Figures 1 and 2). Triglycerides comprised approximately 50% of the fresh weight of the mature, stage 5 embryos. Interestingly, triglycerides tended to be more abundant in the embryos than female gametophytes.

Cultured cells of several plant species were subjected to triglyceride analysis and representative results are shown in Table 1. Embryogenic calli of both Norway spruce and loblolly pine exhibited triglyceride levels dramatically lower than those found in zygotic embryos. Embryogenic conifer calli are characteristically composed of small, dense cells of the somatic embryos along with a high proportion of

large, vacuolated unorganized cells and suspensor cells. Since triglyceride measurements in cells of the somatic embryos might be influenced by the empty, unorganized cells in the calli, individual Norway spruce somatic embryos were isolated, pooled and analyzed. The triglyceride levels in these samples did tend to be higher than in whole callus samples, yet remain far lower than values obtained with zygotic embryos. Low levels of lipids in tissue cultured derived cells are not uncommon. Lipids in morning glory (Ipomoea sp.) and Glycine max unorganized calli were 1/50th the amount found in seeds (Tattrie and Veliky, 1973). Although the fatty acid composition of zygotic and asexual Theobroma cacao embryos were found to be qualitatively similar, the culture-derived embryos had fatty acid levels approximately one-tenth that of the zygotic embryos (Pence et al., 1981). In a study involving conifers, a comparison of callus, seeds and intact tissues of Pinus elliottii also revealed that the fatty acid content of cultured tissue was qualitatively similar to seed, needle and stem tissues (Laseter et al., 1973). "Total extractable lipids" comprised 65% of the seed weight (dry wt.), while callus contained only 4% lipids.

Cells in carrot calli generally contained higher triglyceride levels than cultured conifer cells (Table 1). Notable is the fact that these tissues were unorganized proliferating calli and no effort was made to analyze an enriched sample of somatic embryos. Lipid bodies have been reported to be relatively abundant in proliferating cells of carrot cultures, as judged by ultrastructural examination (Blank et al., 1977).

ABA has been shown to enhance the morphological development and maturation of somatic embryos of a number of plant species (Ammirato, 1974, 1983), including conifers (Dunstan et al., 1988; Von Arnold and Hakman, 1988). In our experiments, ABA treatment led to a significant improvement in the number of developing embryos per Norway spruce callus (Figure 3), embryo production being optimal at ABA concentrations of 10-20 μM. Triglycerides were also affected by ABA concentration. As with embryo production, increasing ABA concentrations led to enhanced triglyceride accumulation, with the response reaching an optimum or plateau near 20 μM ABA. Von Arnold and Hakman (1988) also noted that lipids, visualized by histochemical staining, accumulated in ABA-treated somatic embryos of Norway spruce, but accumulation was never observed in untreated cells.

Storage lipids, triglycerides, are important energy and substrate reserves for zygotic embryo

development and germination. These are obviously energy and substrate demanding processes. Lipid catabolism also plays an important role during organogenesis of Pinus pinaster. Shoot initiation was found to be accompanied by significant reductions of triglycerides, which serve as important reserves during development (Tranvan et al., 1988). It is likely, then, that storage lipids (triglycerides) are also important during somatic embryo development, at least in species having oil-rich seeds. Cultured cells and somatic embryos of many species, including conifers, do not accumulate triglycerides. Cultured tissues also fail to accumulate other reserve substances. The somatic embryos of both rapeseed and cotton have been found to accumulate lower levels of storage proteins than zygotic embryos (Crouch, 1982; Shoemaker et al., 1987). It is possible that the absence of these storage reserves in somatic embryos hinders the late stages of embryo development and germination. It is interesting to note that cultured carrot tissues have higher levels of triglycerides, and fully developed plantlets are remarkably easy to produce from these cultures.

An improvement in the accumulation of triglycerides may facilitate or promote later stages of conifer somatic embryo development and germination. Data in this report suggest that ABA enhances triglyceride accumulation in conifer cells grown in vitro. Growth regulators have also been shown to affect the fatty acid composition of unorganized soybean suspension cultures, although the quantity of lipids was not dramatically changed (Stearns and Morton, 1975). Gibberellins appeared to have a greater effect than the auxins or cytokinins tested (ABA was not used in that study). Other medium components might also influence triglyceride synthesis and accumulation. Both the molar ratios as well as levels of fatty acids in Theobroma cacao embryos could be controlled by the concentration of sucrose in the culture medium (Pence et al., 1981). Compounds serving as substrates of fatty acid and triglyceride biosynthesis might also enhance formation of reserve lipids. These factors will be tested in attempts to enhance embryo development and germination in conifer cultures.

ACKNOWLEDGEMENTS

D. Hanson and J. Wyckoff are thanked for their help in these studies. C. Maynard and T. Walthousen of SUNY at Syracuse and personnel at Westvaco Corporation (Summerville, SC) are acknowledged for collecting and shipping cones used in this study.

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Table 1. Triglyceride levels in culture-derived cells. Values represent the range of triglyceride levels observed in cells originating in a number of trials.

Tissue	[triglyceride] (µg/mg fwt)
Norway spruce embryogenic calli	0.5 - 4
Norway spruce somatic embryos	0.7 - 8
loblolly pine embryogenic calli	0.8 - 4
carrot proliferating calli	3 - 23

Figure 1. Triglyceride levels in developing Norway spruce embryos and female gametophyte tissues.

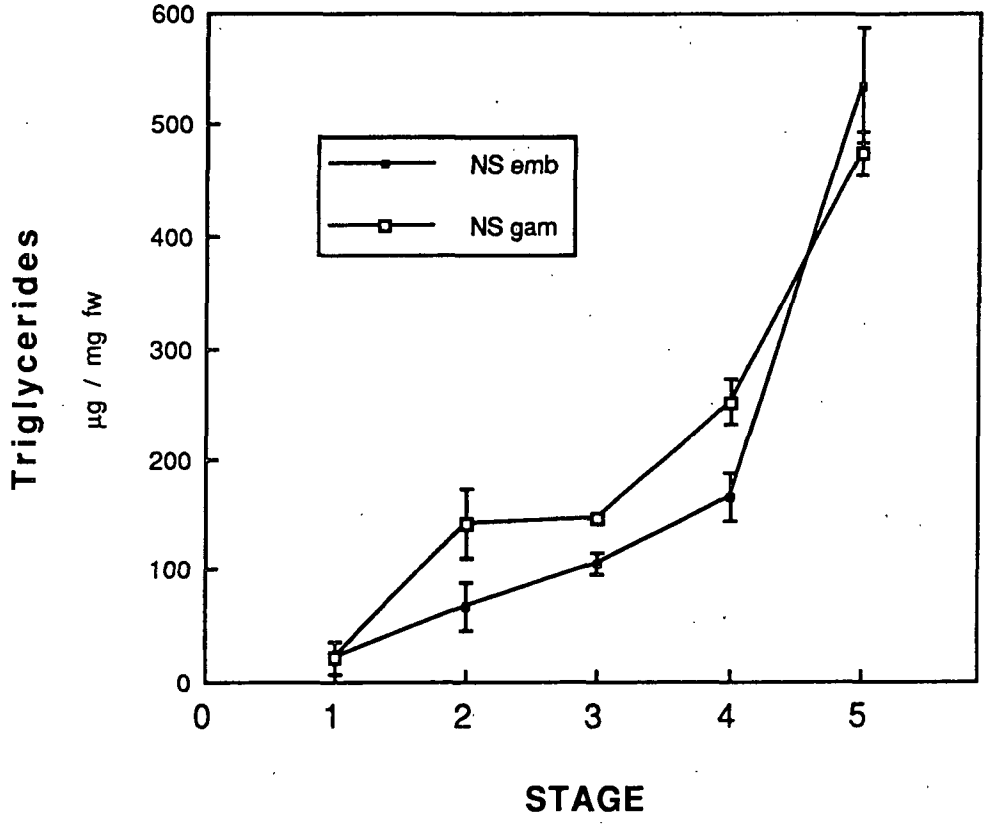

Figure 2. Triglyceride levels in developing loblolly pine embryos and female gametophyte tissues.

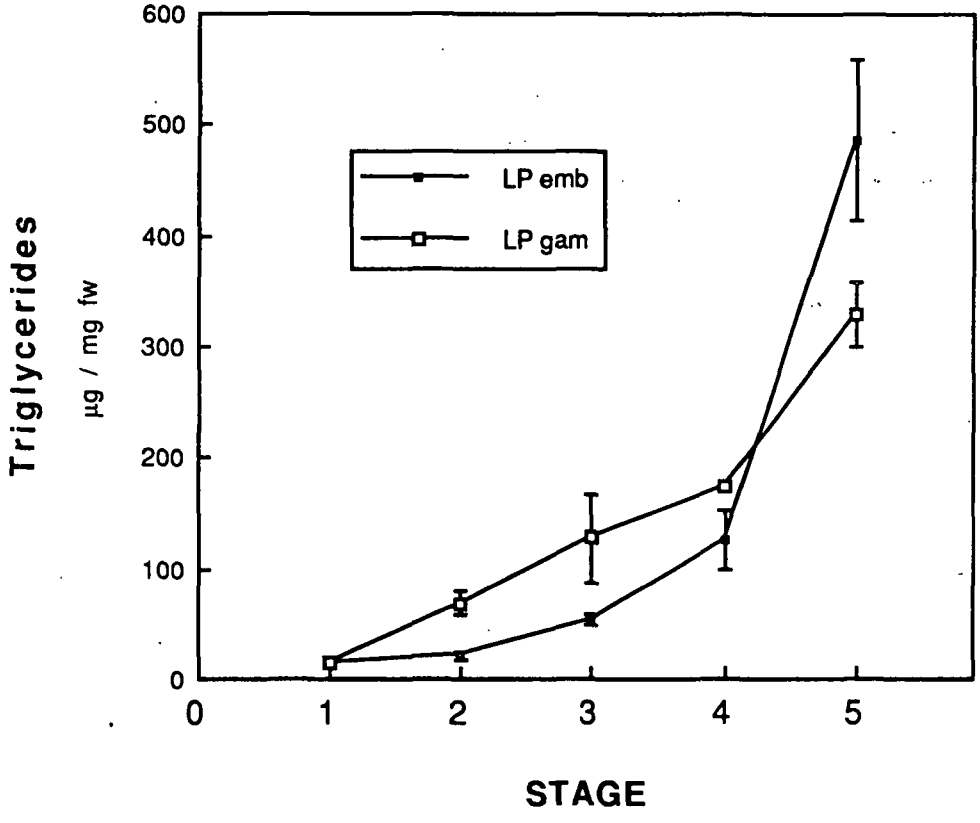

Figure 3. Effect of ABA on embryo production and triglyceride levels in Norway spruce somatic embryos. Somatic embryos having well defined cotyledonary primordia were isolated from the calli, counted and sampled triglyceride analysis. The somatic embryos were similar to stage 3 zygotic embryos.